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EVIDENCE FOR AN ADIPOKINETIC FUNCTION OF THE RPCH ACTIVITY PRESENT IN THE DESERT LOCUST NEUROENDOCRINE SYSTEM[1]

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Crustacean red pigment concentrating hormone (RPCH) is an invertebrate neurosecretory hormone that has been fully characterized and synthesized (Fernlund and Josefsson, 1972), and considerable data are currently available concerning this octapeptide (Josefesson, 1975; Carlsen, Christensen, and Josefsson, 1976; Christensen, Carlsen, and Josefsson, 1977). By contrast, although it has long been known that RPCH-like activity is present in the cephalic neuroendocrine systems of many insects (Hanstrom, 1940; Brown and Meglitsch, 1940; Thomsen, 1943), prior to the initiation of this study little was known of the function or chemistry of the insect material (see Knowles, Carlisle, and Dupont-Raabe, 1955). In view of the availability of synthetic RPCH, the relative ease of the standard *Leander* bioassay for this hormone, and an understanding of RPCH chemistry, it was felt that studies designed to examine the possible function and chemistry of the insect red pigment concentrating activity (RPCA) could be of special interest. Such studies were therefore begun in Copenhagen in the fall of 1975. Data dealing with the presence of RPCA in several insects and the probable function of RPCA in the desert locust *Schistocerca gregaria* are discussed below. These data confirm and extend reports, published after the completion of this study, dealing with the structure and biological activity of the locust adipokinetic hormone (Stone, Mordue, Batley, and Harris, 1976; Mordue and Stone, 1976).

Materials and Methods

Animals

Specimens of *S. gregaria*, reared from eggs provided by the Centre for Overseas Pest Research, London, England, were maintained under crowded conditions in cages with a 16 hr daily photophase and a temperature of about 30° C, and fed frequently on lettuce and bran. Assays for RPCA in fifth instar organs used animals in postmolt (0–1 day after ecdysis), intermolt (4–5 days after ecdysis; no internal apolysis), and premolt (9–10 days after ecdysis; obvious advanced new cuticle formation). Adult organs were assayed 1, 10 and 20 days post-fledging; only the 20 day animals were from actively mating populations. Most of the functional studies used adults of both sexes and all ages; no sexual or age-related

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differences in response were noted. Neck-ligatured specimens of *S. gregaria* were prepared by tightly tying a thin string around the neck just posterior to the head capsule and then cutting off the head just anterior to the ligature.

Specimens of *Tachycines asynamorus* and an unknown *Dytiscus* sp. were collected near Copenhagen. Other insect species were obtained from Danish laboratory cultures (*Tenebrio molitor, Periplaneta americana,* and *Calliphora erythrocephala*), or from the Centre for Overseas Pest Research (*Locusta migratoria*), imported from California (*Danaus plexippus*). These insects were generally adults of mixed ages and sexes. However, both large nymphs and adults of *T. asynamorus,* and a mixture of late premolt fifth instars and newly fledged adults of *L. migratoria* served as donors. Organs from all of these species, except the latter (see below), were collected soon after their arrival in the laboratory. The crustaceans, *Leander adspersus, Crangon vulgaris,* and *Carcinus maenas,* were collected near the Danish coast and maintained for variable periods in artificial sea water at 10° C.

Extractions

Brains and corpora cardiaca (CC) from *S. gregaria* and other insects were normally collected by dissection at intermediate magnification (about 20×), using #5 jeweler's forceps and iridectomy scissors, but dissection of specific regions of these organs in *S. gregaria* used higher magnifications. The pars intercerebralis was roughly separated from the remainder of the brain by cutting a deep V-shaped wedge out of the mid-dorsal region of each brain; cuts were initiated just lateral to each antennal nerve. Great care was used in attempts to separate the glandular region of the CC from the storage lobe (Highnam, 1961), but total separation of these two lobes was, as in other such studies (Goldsworthy and Mordue, 1974), probably not accomplished. Dissections were conducted using chilled insect saline.

For RPCA assays on organs from the early portion of the *S. gregaria* life cycle, previously frozen and lyophilized whole heads of the first three instars and previously frozen brains and CC dissected from fourth instars were used; all organs from these young animals, of undetermined sex and molt cycle stage, were collected at the Centre for Overseas Pest Research and transported frozen to Copenhagen. Similarly, specimens of *L. migratoria* CC were collected in London and transported frozen to the Panum Institute. Dissections were not attempted on *T. molitor* or *C. erythrocephala;* instead, heads were lyophilized and stored at −20° C until extraction. All other extracts were prepared from freshly dissected material.

For the studies on RPCA content during the *S. gregaria* life cycle, extracts of first, second, and third instar heads were made by pulverizing lyophilized material in a small mortar and extracting the powder obtained at a concentration of ten heads/ml (first instar) or five heads/ml (second and third instars). Extracts of fourth instar brains and CC were made at concentrations of four brains/ml and 2 CC/ml. Extracts of fifth instar and adult whole brains and CC were made at 2/ml, and 1/ml, respectively; 4 fifth instar or adult brains or 4 CC were pooled for each extraction. Brain and CC regions were usually extracted at ten brain

regions and 0.5 CC regions/ml. Extracts of other insects used similar techniques, with concentration ranging from 1 CC/ml (*L. migratoria*) to 20 heads/ml (*C. erythrocephala*). For positive controls during the functional studies on *S. gregaria*, CC extracts of various concentrations were lyophilized and the residues dissolved at a concentration of 1 CC/50 μl.

For all extracts, a measured volume of glass-distilled water was placed in a Potter- Elvejem homogenizer and chilled on ice. Either fresh organs rapidly dissected from severed heads, lyophilized powder, or frozen organs were then placed directly into the ice cold solvent. Samples were then immediately homogenized for 1 min at slow speed with a motor-driven pestle, replaced in the ice bath for 1 min, and rehomogenized for 1 min at high speed. Crude extracts were transferred to centrifuge tubes and boiled for 5 min. After boiling, the extracts were centrifuged for 15 min at 12,000×*g* in a Sorvall RC2-B superspeed centrifuge maintained at 4°C. The resulting supernatants were removed and frozen at −20° C until assayed.

Hemolymph for RPCA analysis from selected *S. gregaria* stages was squeezed from a severed rear leg, collected in microcapillary tubes, diluted 1:1 with 1.6% NaCl, boiled 1–3 min, centrifuged for 30 min at 12,000×*g* in the above centrifuge, and the supernatant frozen for subsequent assay.

Assays

RPCA was assayed on eyestalkless specimens of *L. adspersus* using a (2 + 2) point parallel line assay with five animals at each dose (Fernlund, 1968). Synthetic RPCH was used as standard in concentrations of 2.48×10^{-4} and 0.82×10^{-4} μg/ml. This method is referred to below as the *Leander* assay. One unit of RPCH is defined as 0.62×10^{-4} μg of the synthetic hormone.

The effects of RPCH on *S. gregaria* heart rate and malpighian tubule activity were assayed as described by Mordue and Goldsworthy (1969). Total hemolymph lipids were routinely measured by a colorometric method (Goldsworthy, Mordue, and Guthkelch, 1972) using a total serum lipid kit obtained from Boehringer Corp. Ten μl of *S. gregaria* hemolymph (collected into a microcapillary from a small puncture near a rear leg), and 25 μl of *L. adspersus* hemolymph (removed from the pericardial cavity with a Carlsberg micropipette) were routinely used for lipid determinations.

Hormones and analogues

The synthetic RPCH, pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2, used during this study was that mentioned above; it was available at the Panum Institute. The analogues used have recently been synthesized in the same laboratory (Christensen, Carlsen, Josefsson, 1977; Christensen, Carlsen, and Josefsson, in preparation). They include: first, the tyrosine analogue, in which phenylalanine is replaced by tyrosine; secondly, the tetrapeptide, pGlu-Pro-Gly-Trp-NH_2; thirdly, the heptopeptide amide, in which the C-terminal tryptophan amide is removed and glycine amide is the C-terminal; and fourthly, the heptapeptide, with glycine as the C-terminal amino acid.

Hemolymph lipid analysis

In one experiment, a pooled sample of 100 μl hemolymph from several experimental or several control animals was directly added to 1 ml chloroform:methanol 2:1, v/v), and extracted for 1 min. After separation, the lower phase was collected, and the upper extracted again with 500 μl chloroform:methanol (1:2, v/v) for 1 min. After mild centrifugation, the upper phase of this extraction was combined with the previous lower phase. The combination phases were then washed with 300 μl 0.1% NaCl, vortexed 1 min, centrifuged, and the bottom phase collected. Each sample was then evaporated under nitrogen and frozen for subsequent analysis by thin layer chromatography (TLC) and for gravimetric total lipid determination.

TLC was performed on the above extracts using Merck Kiselgel thin layer plates as described by Stahl (1967). Lipids from both experimental and control extracts were applied in volumes containing three concentrations of total lipids, approximately 50, 100, and 200 μg. The solvent system was petroleum ether (bp 60–80°C):ether:acetic acid (70:30:1 v/v). The standards were contained in a TLC reference standard obtained from NU-CHECK Preparations, Elysian, Minnesota, U. S. A. Lipids were visualized with I_2 vapor and spraying with chromic sulfuric acid.

Since TLC showed 1,2-diglycerides to be the major glycerol-containing lipids in the above samples, diglycerides were quantified by the method of Wahlefeld (1974) after lipase hydrolysis.

Some of the data were analyzed by Student's *t*-test; in this report the term significant refers to statistical significance in this test at the 5% level or better. Most of the data is presented as mean ± the standard error of the mean (s.e.m.).

Results

Preliminary experiments

In several initial experiments the *Leander* assay was used to quantify RPCA in heads, brain-CC-corpora allata complexes, and CC of several available insect species. Although all examined species contained some RPCA (see Table 1), variations between species amounted to about four orders of magnitude (*i.e.*, 0.1 to 920 *Leander* units/organ). Two locusts, *S. gregaria* and *L. migratoria,* exhibited the highest RPCA levels, while the lepidopteran *D. plexippus* and the dipteran *C. erythrocephala* contained the least RPCA.

The high level of RPCA in *S. gregaria* CC prompted further preliminary studies on this species. These assays clearly demonstrated RPCA in both CC and brains from adults of variable age and both sexes. In addition to testing these initial extracts in the *Leander* assay, the effects of *S. gregaria* CC extracts on the dark chromatophores of the *C. vulgaris* body, uropods, and telson, and on *C. maenas* erythrophores were also examined. Several assays on each crustacean species clearly demonstrated that *S. gregaria* CC extracts produced dark chromatophore pigment concentration in *C. vulgaris* and erythrophore pigment concentration in *C. maenas.* Comparable experiments dealing with the effects of synthetic RPCH in these two crustaceans (Herman and Josefsson, unpublished) demonstrated that the effect of RPCH was qualitatively identical in both species to that of *S. gregaria* CC extracts. On the basis of these early experiments, and the availability of

TABLE I

RPCA content of various insect species (CA represents corpora allata; CC, corpora cardiaca). All assays were conducted once on extracts of pooled tissues.

Species tested	*Leander* units/organ
Schistocerca gregaria	920/CC
Locusta migratoria	340/CC
Dytiscus sp.	120/CC
Tachycines asynamorus	28/CC
Tenebrio molitor	13/Head
Periplaneta americana	2/CC
Danaus plexippus	0.8/Brain + CC + CA
Calliphora erythrocephala	0.1/Head

reasonable numbers of this well-studied and convenient laboratory animal, *S. gregaria* was chosen for further experimental studies of insect RPCA.

RPCA during the S. gregaria *life cycle*

In subsequent experiments *Leander* assays of the RPCA present in extracts of whole heads of first, second and third instars, brains and CC of fourth instars, and brains, CC, and hemolymph of selected stages of the last instar and adults of both sexes were conducted. Single assays of pooled samples indicated that first, second, and third instar heads contained, respectively, 8, 16, and 310 *Leander* units/head, and that the fourth instar brain and CC contained 0.1 and 250 *Leander* units/organ, respectively. The results obtained from the last two developmental stages are summarized in Table II.

Several points emerge from the consideration of the above data and those of Table II. First, RPCA is present in all post-embryonic life cycle stages. There appears to be a rapid increase in activity during the first three instars (to about 40 times the initial value), followed by a slower increase into the adult stage. The mean combined adult CC RPCA value is about 100 times that found in the first instar, and mean combined adult CC and brain RPCA levels are significantly higher than those found in the fifth instar. Secondly, RPCA is present in both

TABLE II

RPCA content of Schistocerca gregaria *hemolymph, corpora cardiaca, and brains. Data are presented in* Leander *units/organ or per ml hemolymph, with the number in parentheses indicating the number of assays performed on separate pooled samples.*

Stage and sex	Hemolymph	Corpora cardiaca	Brains
Fifth Instar			
Male	—	460 ± 141 (3)	0.4 ± 0.1 (3)
Female	—	337 ± 77 (3)	0.3 ± 0.1 (3)
Combined	1.6 (1)	399 ± 84	0.3 ± 0.1
Adult			
Male	—	827 ± 205 (3)	1.9 ± 0.3 (3)
Female	—	797 ± 106 (3)	0.7 ± 0.3 (3)
Combined	1.0 ± 0.5 (3)	812 ± 115	1.3 ± 0.3

the brain and CC of at least the last three developmental stages. Thirdly, RPCA is present in comparable amounts in the brain and CC of both sexes; the data suggested the possibility of higher RPCA levels in males, but significant sexual differences were not observed. Fourthly, the individual assays showed a range of 440 to 2700 times as much RPCA in the CC as in the brain; RPCA is clearly concentrated in the former organ. Fifthly, RPCA is present in hemolymph from both the final instar and the adult stage. Finally, analysis of data obtained from specific stages of the last instar and the adult stage indicated significant alterations in CC RPCA content during each stage. In the last instar, mean values were 213 ± 19 (N = 2) and 491 ± 97 (N = 4) for, respectively, postmolt and intermolt plus premolt. By contrast, the adult mean value on the day of eclosion was 1120 ± 57 (N = 2), while the mean for 10 and 20 days post-eclosion was 658 ± 106 (N = 4). It therefore appears that CC RPCA increases during the fifth instar and decreases after adult fledging. In adults, the post fledging CC RPCA content decrease at 10 and 20 days was associated with an apparent increase in hemolymph RPCA activity (0.6 *vs.* 1.3 *Leander* units/ml, respectively).

The concentration of RPCA in the CC, the presence of RPCA in the hemolymph, and the apparent variations of RPCA during the life cycle, jointly suggested that RPCA might act as a neurosecretory hormone in *S. gregaria*. Fortunately, several reasonably convenient assays for such hormones in this species had been established by previous research (Goldsworthy and Mordue, 1974). The effects of synthetic RPCH in three of the test systems available were, therefore, examined. Using the techniques described by Mordue and Goldsworthy (1969), the effect of variable amounts of RPCH on heart rate and amaranth excretion were examined. Several attempts to demonstrate an effect of the synthetic hormone in these assays were unsuccessful. In certain experiments minor increases in heart rate and amaranth clearance were obtained, but the results were not significantly different from control values. In view of the relatively striking results with RPCH described below, further experiments with these two assays were not attempted.

RPCH effects on S. gregaria *hemolymph lipids*

Effect of RPCH on adults and immature animals. Initial experiments on hemolymph lipids examined the response of intact adults to injections of synthetic RPCH or *S. gregaria* CC extracts. The results of two separate experiments, using intact adults of both sexes and variable age, showed that both synthetic RPCH and CC extracts caused pronounced elevation of adult hemolymph total lipid levels. The values (in g total lipid/100 ml hemolymph) obtained one hr after injection were: adults injected with 1 μg RPCH in 50 μl distilled water, 4.23 ± 0.33 (N = 7); adults injected with an extract containing the equivalent of one CC in 50 μl distilled water, 4.62 ± 0.28 (N = 7); and adults injected with 50 μl distilled water, 1.17 ± 0.1 (N = 10). Comparable results were obtained in one experiment using neck-ligatured adults. Clearly, RPCH was adipokinetic in the presence or absence of the *S. gregaria* head. In numerous additional experiments, using *S. gregaria* from three separate colonies, elevation of hemolymph lipid was always obtained in response to suitable doses of RPCH or *S. gregaria* CC extracts in either sex or any age of the adult stage.

In two additional experiments the response of intact, mixed sex, intermolt fifth

instars to injections of synthetic RPCH were examined. In the first experiment, animals were injected with hormone, and hemolymph was withdrawn for analysis after 60 min. An apparent response occurred in these animals, since five injected with RPCH, and five injected with distilled water, had total hemolymph levels of 0.47 ± 0.09 and 0.31 ± 0.04 g/100 ml, respectively. Since these results were not significantly different, the experiment was repeated with hemolymph samples taken both before injection and one hr after injection. When the results of this second experiment were calculated on the basis of increase/animal, 0.1 μg RPCH/locust ($N = 7$) caused an increase in hemolymph total lipid of 0.23 ± 0.04 g/100 ml, while distilled water ($N = 7$) led to an increase of 0.10 ± 0.02 g/100 ml. These latter results, although certainly less striking than those obtained with adults, were significantly different; it therefore appears that RPCH is adipokinetic in both immature and adult *S. gregaria*.

Dose-response curve after RPCH injections into intact adults. A further series of experiments examined the dose-response relationship between injected RPCH and adult hemolymph total lipid levels. In these experiments, adults of mixed sexes were injected with synthetic hormone, and the response was measured 1 hr after injection. The results of these studies (see Fig. 1) demonstrated that significant increases are obtainable with about 6 ng synthetic RPCH/animal, and that maximal responses require about 50 ng.

Effects of RPCH analogues in intact adults. In further experiments the effects of synthetic RPCH analogues on hemolymph total lipid levels in intact *S. gregaria*

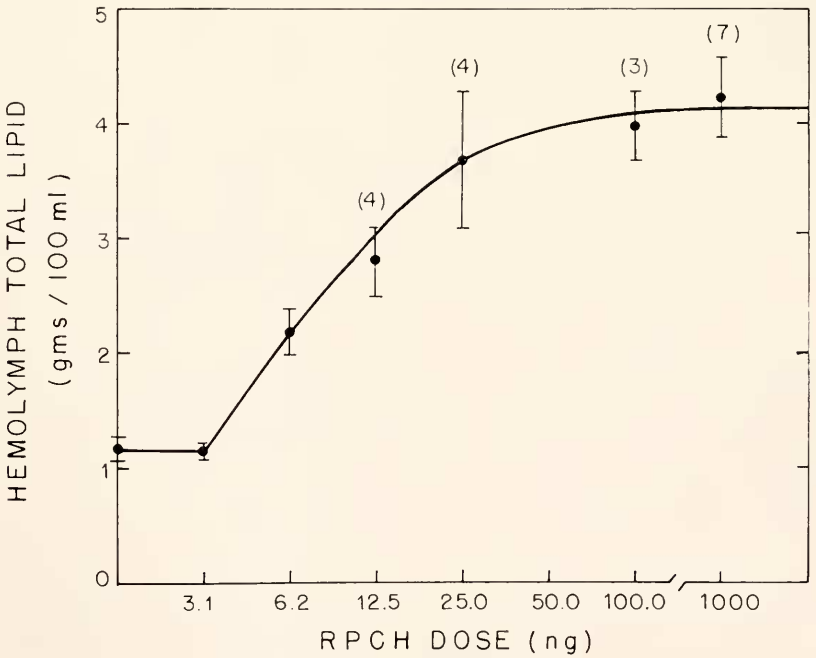

FIGURE 1. Dose-response curve of adipokinetic response of *Schistocerca gregaria* adults injected with synthetic RPCH with N in parentheses.

TABLE III

Effects of synthetic RPCH analogues on adult Schistocerca gregaria *hemolymph lipid. Data are presented as mean ± s.e.m. with N in parentheses. All peptides were injected at a dose of 1 μg/animal, and response was measured one hr after injection. RPCH is the first peptide in the table.*

Peptide tested	Total hemolymph lipid (gm/100 ml)
pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2 (6)	7.69 ± 1.20
pGlu-Leu-Asn-Tyr-Ser-Pro-Gly-Trp-NH_2 (6)	7.32 ± 0.88
pGlu-Pro-Gly-Try-NH_2 (6)	2.43 ± 0.30
pGlu-Leu-Asn-Phe-Ser-Pro-Gly-NH_2 (5)	2.18 ± 0.97
pGlu-Leu-Asn-Phe-Ser-Pro-Gly-OH (5)	1.66 ± 0.27
Distilled Water (6)	1.85 ± 0.29

adults were examined. The results of these studies, summarized in Table III, show that the tyrosine analogue, which has high activity in the *Leander* assay (Christensen *et al.,* in preparation) is apparently equally as effective as RPCH in *S. gregaria.* By contrast the tetrapeptide, which has low RPCH activity (Christensen *et al.,* in preparation), did not result in significant increases, but may have slightly elevated total lipid levels. The heptapeptide and heptapeptide amide, which are inactive in the *Leander* assay, were also without effect in *S. gregaria.* The adipokinetic effect of RPCH is specific to RPCH or structurally similar molecules.

RPCH effects on specific hemolymph lipids. After completion of one of the experiments on intact animals described above, 100 μl of hemolymph from RPCH-injected animals and 100 μl of hemolymph from control animals were collected and subjected to chloroform:methanol extraction, gravimetric determination of total lipid, and TLC analysis.

The results of the gravimetric analysis were comparable to those obtained by the colorimetric method (5.31 *vs.* 4.23 g/100 ml, respectively, for RPCH injected adults and 1.27 *vs.* 1.17 g/ml for controls). The higher value obtained by gravimetric analysis of hemolymph from RPCH-injected animals may be due to an absence of linearity in the colorimetric method at lipid levels over 4.0 g/100 ml (as reported by the manufacturer), or to the fact that the animals injected with RPCH had a somewhat longer time to respond to the injected hormone.

The TLC analysis indicated a substantial increase in hemolymph 1.2-diglyceride, and a possible decrease in both phospholipid and cholesterol in response to RPCH, but revealed no striking changes in other hemolymph lipid classes. Previous research (Mayer and Candy, 1969) had suggested a selective elevation of locust hemolymph 1.2-diglyceride in response to intrinsic adipokinetic hormone. We therefore decided to extend our TLC results by quantification of 1.2-diglyceride levels in hemolymph from both experimental and control animals. On the basis of an average molecular weight of 620 (diolein), the amount of 1.2-diglyceride was found to be 4.20 and 0.33 g/ml hemolymph, respectively, in RPCH-injected and control animals. Based on the above gravimetric analysis of total lipids, 1.2-diglycerides constituted 70% of the total hemolymph lipid in RPCH-injected animals, but only 26% of the total in control animals. These quantitative data, when coupled with our TLC results, clearly demonstrated that 1.2-diglycerides were the major lipids accumulating in *S. gregaria* hemolymph in response to RPCH injections.

RPCH and adipokinetic hormone localization in the neuroendocrine system.

Earlier research had reported the absence of locust adipokinetic hormone in brain (Mayer and Candy, 1969) and the concentration of adipokinetic hormone in the CC glandular lobe (Goldsworthy *et al.*, 1972). In further studies the *Leander* assay was used to quantify RPCA in the pars intercerebralis, brain minus pars intercerebralis, and CC glandular and storage lobes. The data from two such experiments are presented in Table IV.

As the above data show, RPCA was present in all regions examined, with 93% of the total brain RPCA localized outside the pars intercerebralis and 97% of the CC RPCA present in the glandular lobe. In one experiment, RPCA was also found in the corpora allata (0.06 *Leander* units/gland) and in the hypocerebral ganglion (26 *Leander* units/ganglion) of mature adults of mixed sexes. [We believe, in agreement with Thomsen (1943), that the RPCA in the hypocerebral ganglion may be due to the difficulty of separating it completely from the CC. By contrast, the corpora allata, which can be readily separated in this species, apparently contain a low level of RPCA.] The RPCA present in the brain in these experiments was considerably higher than that found in earlier studies (see Table II). We cannot account for this increase with certainty, but it may indicate that extracts of 10 brains/ml (instead of the 2 brains/ml used in Table II) yield more precise results.

In other experiments with *S. gregaria*, adipokinetic activity was assayed in CC glandular and storage lobe extracts. The results of these experiments (see Table V) generally agreed with those reported earlier (Goldsworthy *et al.*, 1972). Significant responses were observed with injections containing the equivalent of 0.4 lobes of each region, but only the glandular lobe extracts produced a significant response at 0.04 lobes/injection. Glandular lobes, therefore, contain at least ten times the adipokinetic activity of storage lobes. Very old mature males and females were used for these experiments; this may account for the high control values.

Absence of RPCH effect on L. adspersus *hemolymph lipids*

The above results suggested that RPCH might also influence hemolymph lipid levels in the shrimp *L. adspersus*. Experiments to test this possibility revealed that total hemolymph lipid levels (in g/100 ml) were: in intact shrimp, 1.94 ± 0.31 ($N = 14$); in eyestalkless shrimp, 1.24 ± 0.35 ($N = 10$); in eyestalkless shrimp

TABLE IV

RPCA localization in adult Schistocerca gregaria *brain and corpora cardiaca.* A and B represent the first and second experiments; pooled tissues are from male and female adults of both sexes used for extracts; and experimental duration = 1 hr.

Organ and region	*Leander* units/region			Mean % of total units
	A	B	Mean	
Brain				
Pars intercerebralis	0.4	0.3	0.4	7
Brain minus pars intercerebralis	4.2	4.4	4.3	93
Corpora cardiaca				
Glandular lobe	760	1350	1055	97
Storage lobe	50	8	29	3

TABLE V

Effect of glandular and storage lobe extracts on adult Schistocerca gregaria *hemolymph total lipids.* Data are presented as mean ± s.e.m. with N in parentheses; controls injected with 50 μl distilled water; and experimental duration = 1 hr.

Amount injected	Total hemolymph lipid (g/100 ml)		
	Controls	Storage lobe	Glandular lobe
0.4 lobes	3.86 ± 0.08 (8)	6.44 ± 0.63 (11)	6.16 ± 0.44 (11)
0.04 lobes	3.73 ± 1.07 (5)	4.73 ± 0.62 (6)	7.40 ± 0.93 (6)

injected with 50 μl 1.6% NaCl, 1.20 ± 0.22 (N = 11); and in eyestalkless shrimp injected with 1 μg RPCH in 50 μl 1.6% NaCl, 1.48 ± 0.21 (N = 14). The data did not therefore indicate an adipokinetic action of RPCH in *L. adspersus.*

DISCUSSION

The results confirm earlier reports of RPCA in representatives of the Orthoptera, Dictyoptera, Coleoptera, Lepidoptera, and Diptera, and add one new species (*D. plexippus*) to the list of insects possessing such activity. These data provide no information as to the absolute amount, or chemical nature, of the RPCA found in these insects. Future studies must determine if the observed species' differences in RPCA levels are due to variable quantities of the same substance or differing specific activities of a variety of substances.

These studies, and some others (Brown and Meglitsch, 1940; Thomsen, 1943), suggest that RPCA could be ubiquitous in insects. However, Hanström (1940) failed to demonstrate RPCA in whole head extracts of several insects, including all examined Thysanura, Dermaptera, Tricoptera, and Hymenoptera, and certain species of Odonata and Lepidoptera. Apparently, RPCA is not uniformly present in insects. The existing data do indicate that the highest RPCA levels routinely occur in Orthoptera. In these studies and those of Hanström (1940) and Thomsen (1943), a total of ten orthopteran species all produced strong responses in RPCH assays.

We have demonstrated RPCA in the cephalic neuroendocrine organs and hemolymph of the last two developmental stages of *S. gregaria* and have shown that significant variations apparently occur in CC RPCA content in the same stages (see Table II). In addition, RPCA can be readily demonstrated in extracts of heads or neuroendocrine organs in earlier stages of the life cycle, and RPCH seems to be adipokinetic in both immature and mature individuals. If RPCA resides in the *S. gregaria* adipokinetic hormone (see below), these findings imply that the adipokinetic endocrine system is present and functional in all stages of the *S. gregaria* life cycle. The following data provide strong evidence that *S. gregaria* RPCA is a substance, closely resembling shrimp RPCH, that functions as the adipokinetic hormone of this locust: first, synthetic RPCH is clearly adipokinetic in both intact and neck-ligatured locusts, and doses of about 6 ng are effective in promoting a significant response (see Fig. 1); secondly, the effect of RPCH is apparently limited to structurally similar peptides, such as the tyrosine analogue, and peptides with little or no RPCH activity in shrimp have little or no adipokinetic activity in *S. gregaria* (see

Table III); thirdly, synthetic RPCH acts in a manner at present indistinguishable from the adipokinetic hormone, *i.e.*, it selectively elevates hemolymph 1,2-glycerides (see Results and Spencer and Candy, 1976); fourthly, both RPCA and the adipokinetic hormone are concentrated in the CC glandular lobe (see Tables IV and V); fifthly, both RPCA and the adipokinetic hormone are apparently present in *S. gregaria*, *L. migratoria*, *P. americana*, and *T. molitor* (see Table I; Goldsworthy *et al.*, 1972; Stone *et al.*, 1976), and the available quantitative data agrees that *S. gregaria* contains more of both substances than *L. migratoria*, which in turn contains more of both substances than *P. americana*. In addition, after the completion of this research, two reports appeared dealing with the relationship of RPCH and the locust adipokinetic hormone. One paper presented evidence that injection of crustacean eyestalk extracts elevated locust hemolymph lipid levels, while pure locust adipokinetic hormone produced RPCH-like effects on the chromatophores of several crustaceans (Mordue and Stone, 1976). The other contained a proposed structure for locust adipokinetic hormone, indicating that RPCH and the adipokinetic hormone contain the same six amino acids in identical positions (Stone *et al.*, 1976). In view of the available information, the only reasonable conclusion seems to be that locust adipokinetic hormone and crustacean RPCH are biologically and structurally similar molecules.

It is of interest to compare the quantitative aspects of this study with those of Stone and her colleagues (1976). We found RPCA equal to about 50 ng RPCH/adult CC (mean value of 812 *Leander* units/adult CC in Table II, times 0.62×10^{-4} μg RPCH/*Leander* unit). In addition, the lowest effective dose in our adipokinetic assay was about 6 ng, and the dose required for maximal elevation of hemolymph lipid was about 50 ng (see Fig. 1). By contrast, Stone and coworkers reported 500–800 ng pure adipokinetic hormone/adult CC (glandular lobe only), a minimum effective dose of about 1 ng, and a dose of about 12 ng to be required for a maximal response. Adipokinetic hormone, therefore, appears to have some 6–10% of the activity of RPCH in the *Leander* assay, while RPCH is about 20% as effective as pure adipokinetic hormone when assayed on *S. gregaria*.

We have demonstrated significant RPCA in *S. gregaria* brains, but previous workers failed to find evidence for a locust brain adipokinetic hormone (Mayer and Candy, 1969). Our experiments suggest that failure to demonstrate brain adipokinetic hormone may be explained on the basis of assay sensitivity. The *S. gregaria* adult brains assayed (see Table II) contained a mean value of 1.3 *Leander* units/brain. This amount of RPCH (about 0.08 mg) is about 1% of that required per animal for a significant response in our adipokinetic assay (see Fig. 1), but it is considerably higher than that used for the lowest standard (4 pg/shrimp) in the *Leander* assay. If, as discussed above, pure adipokinetic hormone is equivalent to 10–15 times the RPCA measured in the *Leander* assay, this would mean that each brain contains no more than 1–2 ng pure adipokinetic hormone. Since previous studies (Mayer and Candy, 1969) apparently injected the equivalent of 0.1 brain/locust, and a significant response in the adipokinetic assay requires 1–2 ng pure adipokinetic hormone (Stone *et al.*, 1976), it is not surprising that brain adipokinetic hormone activity was not observed. (A similar conclusion is reached if the higher values for brain RPCA of Table IV are used.)

The data (Table IV) suggest that RPCA is manufactured in the pars inter-

cerebralis, accumulates in some other region of the brain, and is eventually transported to the CC storage lobe. This interpretation would account for the low pars intercerebralis RPCA, and the higher RPCA levels in the remainder of the brain and in the storage lobe. The presence of RPCA in all brains assayed indicates that storage lobe RPCA comes from the brain; its presence in storage lobe extracts is probably not due to attached portions of the glandular lobe. In addition, RPCA certainly accumulates in the CC glandular lobe. It may be that the glandular lobe RPCA originates in the brain or storage lobe; the histology of the *S. gregaria* CC is compatible with this possibility (Highnam, 1961). An alternative explanation would be the existence of two RPCA substances, one produced by the pars intercerebralis and the other by the glandular lobe intrinsic cells. The significance of the presence of RPCA in the *S. gregaria* CA remains to be determined; Thomsen (1943) found low RPCA activity in the CA of several species.

The chromatophorotropic function of RPCH, the hormone-like action of RPCH in *S. gregaria,* the presence of RPCA in a large number of different arthropods, and the apparent structural similarity between RPCH and the locust adipokinetic hormone, jointly suggest that several activities attributed to arthropod neurosecretory hormones could be mediated by a family of RPCH-like peptides. It will therefore be of great interest to characterize and synthesize RPCH-like peptides from different sources, and to investigate the hormonal action of these substances in a variety of arthropod species. Such data could lead to major advances in understanding the evolution of arthropod (or invertebrate) hormones, specifically, and the evolution of peptide hormones generally. It would also provide insight into the feasibility of using substances with RPCH-like activity, or chemicals interfering with such activity, for purposes of arthropod regulation. Patently, if RPCH-like peptides act hormonally in a diversity of arthropods, attempts to regulate arthropod populations by manipulations of systems mediated by RPCH-like molecules would require thoughtful consideration.

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Summary

1. Red pigment concentrating hormone-like activity (RPCA) has been demonstrated and quantified in the head or cephalic neuroendocrine system of several insects. *Schistocerca gregaria* adults contained the highest levels of RPCA.

2. RPCA was found in all post-embryonic life cycle stages of *S. gregaria,* and seems to be present in equal quantities in both males and females. Adult locusts contain significantly more RPCA than do immature animals.

3. RPCA is concentrated in the locust CC glandular lobe but is also present in the CC storage lobe, brain and hemolymph of both mature and immature animals.

Significant variations in CC RPCA content have been demonstrated in both mature and immature stages.

4. Synthetic red pigment concentrating hormone (RPCH) is adipokinetic in both intact and neck-ligatured *S. gregaria* adults and in intact immature animals. Doses of about 6 ng and 50 ng, respectively, result in minimal and maximal adipokinetic responses. RPCH selectively elevates hemolymph 1,2-diglycerides.

5. RPCH in large doses was without effect on crustacean hemolymph lipid levels.

6. Evidence favoring the biological and structural similarity of RPCH and locust adipokinetic hormone is discussed.

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